'ENT COOPERATION TRE. /

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 12 August 1999 (12.08.99)	
International application No. PCT/US98/27364	**Applicant's or agent's file reference** 2115S01334PO
International filing date (day/month/year) 23 December 1998 (23.12.98)	**Priority date** (day/month/year) 23 December 1997 (23.12.97)
Applicant NABEL, Gary, J. et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

23 July 1999 (23.07.99)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Lazar Joseph Panakal
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 2115S01334PO•	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US98/27364	International filing date *(day/month/year)* 23 DECEMBER 1998	Priority date *(day/month/year)* 23 DECEMBER 1997
International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet.		
Applicant THE REGENTS OF THE UNIVERSITY OF MICHIGAN		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets.

   [X] This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 0 sheets.

3. This report contains indications relating to the following items:

   - I [X] Basis of the report
   - II [ ] Priority
   - III [ ] Non-establishment of report with regard to novelty, inventive step or industrial applicability
   - IV [ ] Lack of unity of invention
   - V [X] Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI [ ] Certain documents cited
   - VII [ ] Certain defects in the international application
   - VIII [ ] Certain observations on the international application

Date of submission of the demand	Date of completion of this report
23 JULY 1999	17 DECEMBER 1999
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer LAURIE SCHEINER Telephone No. (703) 308-0196

Form PCT/IPEA/409 (cover sheet) (January 1994)★

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US98/27364

I. Basis of the report

1. This report has been drawn on the basis of (*Substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments*):

[X] the international application as originally filed.

[X] the description, pages 1-19 , as originally filed.
pages NONE , filed with the demand.
pages NONE , filed with the letter of _____.
pages _____ , filed with the letter of _____.

[X] the claims, Nos. 1-26 , as originally filed.
Nos. NONE , as amended under Article 19.
Nos. NONE , filed with the demand.
Nos. NONE , filed with the letter of _____.
Nos. _____ , filed with the letter of _____.

[X] the drawings, sheets/~~fig~~ 1-12 , as originally filed.
sheets/~~fig~~ NONE , filed with the demand.
sheets/~~fig~~ NONE , filed with the letter of _____.
sheets/~~fig~~ _____ , filed with the letter of _____.

2. The amendments have resulted in the cancellation of:

[X] the description, pages NONE.

[X] the claims, Nos. NONE.

[X] the drawings, sheets/~~fig~~ NONE.

3. [ ] This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the ~~Supplemental Box~~ Additional observations below (Rule 70.2(c)).

4. Additional observations, if necessary:

NONE

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US98/27364

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. STATEMENT

Novelty (N)	Claims	1-26	YES
	Claims	NONE	NO
Inventive Step (IS)	Claims	1-26	YES
	Claims	NONE	NO
Industrial Applicability (IA)	Claims	1-26	YES
	Claims	NONE	NO

2. CITATIONS AND EXPLANATIONS

Claims 1-26 meet the criteria set out in PCT Article 33(2)-(4), because the prior art does not teach or fairly suggest that expressed Ebola glycoprotein genes may be employed as vaccines. Moreover, prophylactic therapy, as in a method of vaccination is not envisaged by Sanchez et al.

---------- NEW CITATIONS ----------

NONE

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

CLASSIFICATION:

The International Patent Classification (IPC) and/or the National classification are as listed below:

IPC(7): A61K 39/12, 45/00, 39/145, 39/155, 39/205 and US Cl.: 424/199.1, 204.1, 209.1, 211.1, 224.1, 278.1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/27364

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 39/12, 45/00, 39/145, 39/155, 39/205

US CL :424/199.1, 204.1, 209.1, 211.1, 224.1, 278.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/199.1, 204.1, 209.1, 211.1, 224.1, 278.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PALESE, P. et al. Negative-Strand RNA Viruses: Genetic Engineering and Applications Proc. Natl. Acad. Sci. USA October 1996, Vol. 93, pages 11354-11358, see entire document	1-26
Y	SANCHEZ, A. et al. The Virion Glycoproteins of Ebola Viruses are Encoded in Two Reading Frames and are Expressed Through Transcriptional Editing Proc. Natl. Acad. Sci. USA. April 1996, Vol. 93, pages 3602-3607, see entire document.	1-26

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 20 APRIL 1999	Date of mailing of the international search report 10 MAY 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer LAURIE SCHEINER Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

UMV-1334PC

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To: DEANN F. SMITH
LAHIVE & COCKFIELD LLP
28 STATE STREET
BOSTON, MASSACHUSETTS 02109

PCT

NOTIFICATION OF TRANSMITTAL OF INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of Mailing *(day/month/year)*	20 JAN 2000
Applicant's or agent's file reference: 2115S01334PO	IMPORTANT NOTIFICATION

International application No.	International filing date *(day/month/year)*	Priority Date *(day/month/year)*
PCT/US98/27364	23 DECEMBER 1998	23 DECEMBER 1997

Applicant
THE REGENTS OF THE UNIVERSITY OF MICHIGAN

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices)(Article 39(1))(see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

ENTERED
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LAHIVE & COCKFIELD
DOCKET DEPT.
JAN 25 2000
RETRIEVED: 1/26/00
FORWARDED: NFB 1/28

Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer LAURIE SCHEINER
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/IPEA/416 (July 1992)★

VDFS

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

To: DEANN F. SMITH
HARNESS, DICKEY & PIERCE, P.L.C.
P.O. BOX 828
BLOOMFIELD HILLS, MI 48303

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

	Date of Mailing *(day/month/year)* 10 MAY 1999
Applicant's or agent's file reference 2115S01334PO	FOR FURTHER ACTION See paragraphs 1 and 4 below
International application No. PCT/US98/27364	International filing date *(day/month/year)* 23 DECEMBER 1998
Applicant THE REGENTS OF THE UNIVERSITY OF MICHIGAN	

1. [X] The applicant is hereby notified that the international search report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the international search report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] With regard to the protest against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after 18 months from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in rules 90 *bis* 1 and 90 *bis* 3, respectively, before the completion of the technical preparations for international publication.

Within 19 months from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within 20 months from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer LAURIE SCHEINER
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 2115S01334PO	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
International application No. PCT/US98/27364	International filing date *(day/month/year)* 23 DECEMBER 1998	(Earliest) Priority Date *(day/month/year)* 23 DECEMBER 1997
Applicant THE REGENTS OF THE UNIVERSITY OF MICHIGAN		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 2 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. [ ] **Certain claims were found unsearchable** (See Box I).

2. [ ] **Unity of invention is lacking** (See Box II).

3. [ ] The international application contains disclosure of a **nucleotide and/or amino acid sequence listing** and the international search was carried out on the basis of the sequence listing
   - [ ] filed with the international application.
   - [ ] furnished by the applicant separately from the international application,
     - [ ] but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.
   - [ ] transcribed by this Authority.

4. With regard to the title,
   - [X] the text is approved as submitted by the applicant.
   - [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,
   - [X] the text is approved as submitted by the applicant.
   - [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is:

   Figure No. _____
   - [ ] as suggested by the applicant.
   - [ ] because the applicant failed to suggest a figure.
   - [ ] because this figure better characterizes the invention.
   - [X] None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/27364

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 39/12, 45/00, 39/145, 39/155, 39/205
US CL :424/199.1, 204.1, 209.1, 211.1, 224.1, 278.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/199.1, 204.1, 209.1, 211.1, 224.1, 278.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PALESE, P. et al. Negative-Strand RNA Viruses: Genetic Engineering and Applications Proc. Natl. Acad. Sci. USA October 1996, Vol. 93, pages 11354-11358, see entire document	1-26
Y	SANCHEZ, A. et al. The Virion Glycoproteins of Ebola Viruses are Encoded in Two Reading Frames and are Expressed Through Transcriptional Editing Proc. Natl. Acad. Sci. USA. April 1996, Vol. 93, pages 3602-3607, see entire document.	1-26

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 20 APRIL 1999	Date of mailing of the international search report 10 MAY 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT	Authorized officer LAURIE SCHEINER

NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing f amendments under Articl 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more d tailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule" and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions, respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Preliminary Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where Applicable, Article 41.

When ? Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments ?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How ? Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments ?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 11354–11358, October 1996
Colloquium Paper

This paper was presented at a colloquium entitled "Genetic Engineering of Viruses and of Virus Vectors," organized by Bernard Roizman and Peter Palese (Co-chairs), held June 9–11, 1996, at the National Academy of Sciences in Irvine, CA.

Negative-strand RNA viruses: Genetic engineering and applications

PETER PALESE*, HONGYONG ZHENG, OTHMAR G. ENGELHARDT, STEPHAN PLESCHKA, AND ADOLFO GARCÍA-SASTRE

Department of Microbiology, Mount Sinai School of Medicine, 1 Gustave L. Levy Place, New York, NY 10029

ABSTRACT **The negative-strand RNA viruses are a broad group of animal viruses that comprise several important human pathogens, including influenza, measles, mumps, rabies, respiratory syncytial, Ebola, and hantaviruses. The development of new strategies to genetically manipulate the genomes of negative-strand RNA viruses has provided us with new tools to study the structure–function relationships of the viral components and their contributions to the pathogenicity of these viruses. It is also now possible to envision rational approaches—based on genetic engineering techniques—to design live attenuated vaccines against some of these viral agents. In addition, the use of different negative-strand RNA viruses as vectors to efficiently express foreign polypeptides has also become feasible, and these novel vectors have potential applications in disease prevention as well as in gene therapy.**

DNA-Containing Viruses

Among animal viruses, DNA-containing viruses were the first to become amenable to genetic engineering techniques. This breakthrough was achieved for simian virus 40 when a cloned cDNA copy was transfected into cells, resulting in the formation of infectious virus (see Table 1). Transfected mutated cDNA molecules gave rise to defined mutant viruses (1). A second methodology involving the use of homologous recombination allowed, for the first time, the rescue of large DNA-containing viruses such as herpes viruses (2). In this approach, intact herpes viral DNA as well as cloned DNA flanked by viral sequences was transfected into cells. Homologous recombination between the cloned DNA and the wild-type genome can occur, and novel viruses can be selected under appropriate conditions. For example, recombinants with DNA fragments containing a viral thymidine kinase gene can be selected in appropriate cell lines and media, and viruses lacking a thymidine kinase can be isolated in the presence of nucleoside analogs (e.g., Ara T). This general technique allows the successful construction of viral variants of herpes viruses, and similar procedures have been developed for pox viruses (3, 4) and other DNA-containing viruses including adenoviruses (5) and parvoviruses (6). Finally, strategies have been developed to generate infectious as well as mutant viruses by transfecting cosmids containing overlapping portions of large viral genomes. Viruses arise via recombinati n between the cosmids. This system was successfully used to rescue infectious herpes simplex I viruses (7), cytomegaloviruses (8) and Epstein–Barr viruses (9) from their respective cosmids.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Positive-Strand RNA Viruses

RNA-containing viruses belong to a variety of families with diverse replication strategies. Unique among the RNA viruses are the retroviruses, whose replication involves a double-stranded DNA phase, making these viruses an easy target for genetic manipulation. Transfection of full-length cDNA molecules leads to the establishment of replicating virus particles and integration of the viral genetic information into the host genome (10). The engineering of retroviral genomes has become one of the most successful genetic approaches in modern virology and is central to the study both of viral gene expression and of protein structure–function analysis. In addition, retrovirus constructs are among the most widely used vectors for gene transfer and gene therapy (11).

Most of the other positive-strand RNA viruses are also amenable to genetic engineering approaches (Table 1). In the case of the small and medium sized positive-strand RNA viruses, full-length genomic RNA has been shown to be infectious when transfected into cells. Plus-strand RNA serves as mRNA for the synthesis of viral proteins as well as template for viral RNA replication. Thus, transfection of cloned DNA of poliovirus RNA (or of cDNA-derived RNA) into permissive cells results in the formation of infectious virus particles (12).

Remarkably successful have been studies using Sindbis viruses and Semliki forest virus (13, 14). The cDNA-derived RNAs of these positive-strand RNA viruses can be used to efficiently rescue infectious viruses, thus allowing an extensive analysis of the promoter elements of the viral RNAs as well as structure–function studies of the viral proteins. Furthermore, these viruses have received increased attention because of their potential for expressing copious amounts of heterologous genes via recombinant constructs. Up to 10^8 molecules of heterologous protein per cell have been expressed using these systems.†

Introduction of cDNA-Derived RNA into a Negative-Strand RNA Virus (Influenza Virus)

The life cycle of negative-strand RNA viruses differs from that of the other RNA viruses in many ways. Specifically, the genomic RNA of negative-strand RNA viruses is not infectious, and infectious virus particles must also deliver their own RNA-dependent RNA polymerase into the infected cell to start the first round of virus-specific mRNA synthesis.

Thus, approaches different from those used for positive-strand RNA viruses had to be developed to allow the rescue of

Abbreviations: RNP, ribonucleoprotein; HA, . . magglutinin; NA, neuraminidase; VSV, vesicular stomatitis virus.

*To whom reprint requests should be addressed. e-mail: ppalese@smtplink.mssm.edu.

†Belli, B. A., Polo, J. M., Driver, D. A., Latham, E., Banks, T. A., Chang, S. M. W. & Dubensky, T. W., Jr., National Academy of Sciences Colloquium on Genetic Engineering of Viruses and of Virus Vectors, June 9–11, 1996, Irvine, CA, no. 1. (abstr.).

Table 1. Genetic engineering of animal viruses

Type of genome	Prototype viruses	Strategies
dsDNA	Simian virus 40, herpes, adenovirus, poxvirus	Transfection of cDNA; homologous recombination using cloned DNA and intact viral DNA or helper viruses; transfection of cosmids containing viral genes
ssDNA	Adeno-associated virus (AAV)	Transfection of plasmids containing AAV genes
ssRNA	Retrovirus	Transfection of infectious cDNA
	Plus-sense RNA	
	Picornavirus, Semliki forest virus, Sindbis virus	Transfection of cDNA-derived infectious RNA
	Minus-sense RNA	
	Influenza virus, rhabdovirus, parainfluenza virus, bunyavirus	Transfection of reconstituted ribonucleoprotein in the presence of helper virus; rescue of virus from cDNA clones transcribed *in vitro* or *in vivo* in the presence of helper virus or of viral polymerase proteins expressed intracellularly in trans
dsRNA	—	—

ds, Double stranded; ss, single stranded.

genetically engineered viruses of these virus families (Table 1). Site-specifically altered influenza viruses were first obtained by reconstituting *in vitro* a biologically active ribonucleoprotein complex (made of synthetic RNA and purified nucleoprotein and polymerase proteins) and then transfecting the complex into helper virus-infected cells (Fig. 1) (15). The helper virus provides in trans the viral proteins required for amplification of the synthetic RNP complex. Subsequent reassortment of the synthetic gene and helper virus-derived RNA segments, followed by selection for the reassortant (transfectant) virus, allows the introduction of site-specific changes into the genome of influenza viruses (16). Selection of the transfectant virus can be achieved by choosing host range or temperature-sensitive mutants as helper viruses. Alternatively, antibody preparations specific for the viral surface proteins can be used to select against the helper virus or for these novel viral constructs. Following such protocols, six of the eight genes [PB2, hemagglutinin (HA), neuraminidase (NA), NP, M and NS] of influenza A viruses and the HA of an influenza B virus have now successfully been altered by genetic engineering methods (17–22).

Plasmid-Based Reverse Genetics System for Influenza Virus

A method was recently developed to reconstitute a biologically active influenza virus RNP complex within a cell rather than *in vitro*. This alternative approach avoids the need to purify viral proteins and to transfect an RNA–protein complex into cells; instead, this method involves the transfection of plasmids. The first plasmid contains a human polymerase I promoter and a hepatitis delta virus-derived ribozyme sequence which flank the synthetic influenza virus gene. The polymerase I-driven plasmid is cotransfected into human cells with polymerase II-responsive plasmids expressing in trans the viral PB1, PB2, PA, and NP proteins. Such a system involving the use of five plasmids allows the amplification and expression of a synthetic influenza virus gene and takes advantage of the convenience of plasmid transfections as compared with RNP transfections (23). Using this approach, it was possible t rescu a synthetic NA gene into a recombinant influenza A virus. A synthetic HA gene has also been rescued by this novel technique (Fig. 2) (A.G.-S., unpublished results). It should be noted, however, that this plasmid-based reverse genetics system still relies n the presence of a helper virus which provides the genetic backbone int which the plasmid-derived gene can be introduced.

Chimeric Influenza Viruses Expressing Foreign Epitopes or Polypeptides

The development of methods to rescue synthetic RNAs into the genomes of influenza viruses allowed the construction of chimeric viruses expressing a variety of foreign epitopes. Specifically, epitopes derived from HIV, plasmodia, or lymphocytic choriomeningitis virus proteins were successfully expressed in either the HA or the NA of different influenza viruses (16, 24). Such constructs were shown to induce a potent B-cell and/or T-cell response against the foreign epitope in experimental animal systems. Specifically, Li *et al.* (25) gen-

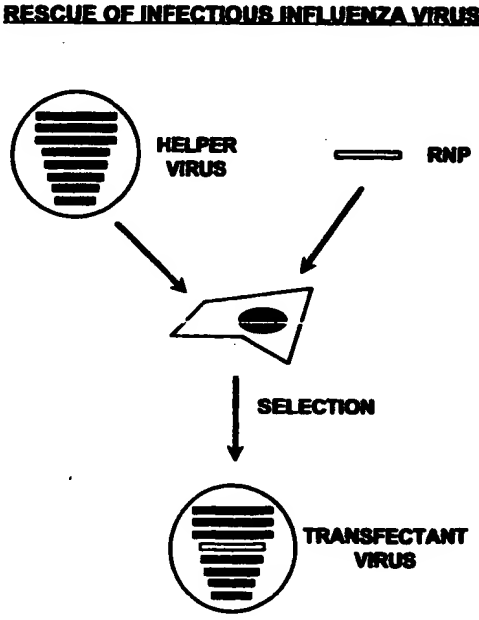

FIG. 1. A reverse genetics system for the rescue of infectious influenza viruses containing cDNA-derived RNA. The method allows the substitution of one of the eight genomic RNA segments of the virus by a synthetic RNA. A biologically active viral ribonucleoprotein complex (RNP) is made *in vitro* by mixing cDNA-derived RNA with purified viral nucleoprotein and polymerase proteins. The RNPs are transfected into cells which have been previously infected with an influenza helper virus. Using a selection method, viruses containing the genetically engineered RNP (transfectant viruses) can be isolated.

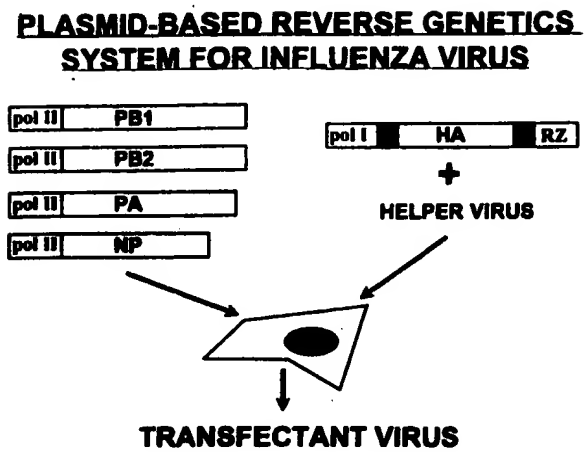

FIG. 2. A plasmid-based reverse genetics system for the rescue of infectious influenza viruses containing a genetically engineered segment. Cells are transfected with four plasmids that are able to express the viral NP and polymerase (PB2, PB1, and PA) proteins from a cellular polymerase II-responsive promoter (pol II). An additional plasmid which contains, for example, the HA open reading frame flanked by the 5′ and 3′ noncoding regions of the viral RNA segment (black boxes) is cotransfected. The HA plasmid is able to express an HA-specific viral RNA by transcription from a polymerase I-responsive promoter (pol I) followed by the ribozyme (RZ)-mediated cleavage of the transcript. The HA-specific RNA segment is intracellularly complexed with the NP and polymerase proteins to form RNPs that can be rescued into a transfectant virus if the cells are also infected with an influenza helper virus. Selection of the transfectant viruses can be performed by using neutralizing antibodies against the HA protein of the helper virus.

erated a recombinant influenza virus that expressed a $CD8^+$ T-cell epitope derived from the circumsporozoite (CS) protein of *Plasmodium yoelii* in its HA. Mice immunized with this transfectant virus made a vigorous cytotoxic T lymphocyte response against this epitope (25). By boosting mice with a recombinant vaccinia virus expressing the CS protein, it was possible to achieve protective immunity (60%) against challenge with live *P. yoelii* sporozoites. Additional protective immune responses were generated by immunizing mice with transfectants expressing B-cell-specific epitopes located in the repeat region of the CS protein of *P. yoelii*. Up to 80% of immunized mice were immune to challenge with one hundred *P. yoelii* sporozoites (26).

Foreign epitopes can be inserted into several sites on the HA molecule of influenza viruses, and most conveniently into the stalk region of the NA. In fact, stretches of more than 80 foreign amino acids have been successfully inserted into the stalk region of the NA (27, 28) (S. Itamura, personal communication). Although some of these constructs show interesting biological properties, this approach of epitope grafting has its limitations in terms of the size and the nature of the epitope that can be expressed (since the chimeric protein may affect the viability of the recombinant virus).

A generic approach to the expression of foreign proteins is the construction of bicistronic genes which can be packaged into infectious particles. The foreign gene can replace the open reading frame of one of the influenza virus genes and the respective influenza virus protein is then translated from an internal ribosome entry site (IRES element) on the genetically engineered gene. Alternatively, the foreign protein can be translated from an internal IRES sequence. Expression of several foreign polypeptides was achieved in this way (16, 29). However, many constructs did not result in viable viruses (unpublished results). Attempts are currently being made to identify the factors which determine the limitations of this approach.

The second method for the expression of foreign proteins takes advantage of autoproteolytic elements placed within a fusion protein. For example, a virus was constructed that expresses a fusion protein consisting of the full-length chloramphenicol acetyltransferase (CAT) protein, the 2A protease of foot and mouth disease virus, and the viral NA (30). This virus was stably passaged and expressed copious amounts of CAT protein in infected cells. However, in all cases of the fusion protein constructs, the foreign protein contains a 16-amino acid extension derived from the 2A protease which may alter the biological properties of the foreign protein.

Rescue of Infectious Rabies Virus from cDNA

Like the segmented negative-strand RNA viruses, the Mononegavirales group packages its own RNA-dependent RNA polymerase into virus particles to initiate viral RNA synthesis. Thus, naked RNA alone is unable to drive the replication cycle. Several approaches were taken to rescue model and full-length RNAs. First, a Sendai virus-like RNA transcript was amplified and expressed by transfecting the naked model RNA into Sendai virus-infected cells (31). This experiment suggests that complementation in trans by the viral polymerase complex is required for the amplification and expression of the viral RNA-like reporter gene. Subsequently, in a remarkable study, Schnell *et al.* (32) succeeded in constructing a plasmid that expresses a full-length rabies virus RNA transcript from a T7 RNA polymerase promoter. The plasmid DNA containing this viral insert was transfected into cells infected with a recombinant vaccinia virus expressing the T7 polymerase. Three other plasmids expressing the rabies virus N, P and L proteins were also cotransfected into these cells. In this recombinant vaccinia virus-driven system, the presence of the viral polymerase complex and of a full-length viral RNA (in plus sense) led to the formation of recombinant rabies virus.

This system has been elegantly exploited to study the promoter elements of rabies virus RNA and to elucidate the interaction of this interesting virus with cells (33). Surprisingly, cells infected with a mutant lacking the virus' only glycoprotein (G) were still able to bud from the cell surface, albeit at a 30-fold lower efficiency (34). This experiment revealed that the surface protein G exhibits an intrinsic exocytotic activity. The system was further developed to show that a hybrid G/HIV-1 glycoprotein was able to form pseudotypes with the "G-less" particle, thus changing the host range by restricting infection to $CD4^+$ cells. This experiment clearly demonstrates that genetic engineering can redirect the host range and cell tropism of rabies viruses. This should prove helpful for the development of novel vaccines as well as for gene therapy.

Rescue of Other Nonsegmented Negative-Strand RNA Viruses

An effective DNA transfection system has also been developed for another rhabdovirus, vesicular stomatitis virus (VSV) (35, 36) (Fig. 3). Again, the polymerase complex (N, P, and L proteins) was expressed in cells from plasmids transcribed by a T7 RNA polymerase-containing vaccinia virus recombinant. Recombinant VSVs expressing an additional transcriptional unit were rescued and high-expression levels of heterologous proteins were achieved (37). In a dramatic experiment, the authors were able to construct a recombinant VSV expressing the CD4 protein. This protein was packaged at levels of up to 30% of the G protein itself, and the recombinant particle had an 18% greater length than wild-type virus due to the extra gene. These results illustrate that VSV is an effective vector to express foreign proteins at high levels, and that the virus is tolerant to the insertion of novel transcriptional units. Reverse genetics systems have also been developed for paramyxoviruses. In the case of measles virus, a cell line constitutively

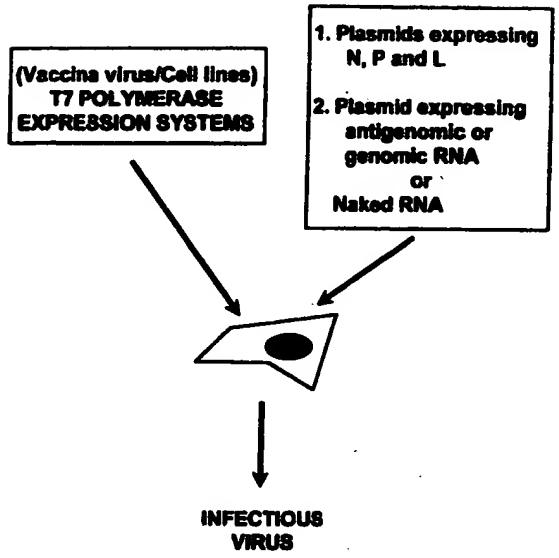

FIG. 3. Reverse genetics systems for the rescue of infectious nonsegmented negative-strand RNA viruses from cDNA. Transcriptionally competent viral RNPs are made by cellular expression of the viral proteins N, P, and L. This can be achieved by a variety of methods, including vaccinia virus-driven expression and/or complementing cell lines constitutively expressing T7 polymerase and viral proteins. The full-length viral RNA can be provided by transfecting plasmids expressing antigenomic or genomic RNA or by directly transfecting naked RNA (plus-sense or minus-sense). The intracellularly assembled RNPs are transcribed and replicated by the viral polymerase complex (N, P, and L proteins) generating infectious viruses.

expressing T7 polymerase and the measles N and P proteins has been used for the rescue of infectious virus from full-length clones (38) and vaccinia virus-based systems have allowed the rescue of respiratory syncytial virus (39) and of Sendai viruses (40, 41).

Most of the earlier systems developed for the nonsegmented viruses used the intracellular expression of antigenomic plus-sense RNA as the template to initiate the replication cycle. Either the plus-sense RNA was transcribed by the T7 polymerase expressed by a vaccinia-recombinant virus (32, 35, 36, 39–41), or transcription was driven by the T7 polymerase which was permanently expressed in cells (38). Recently, an important series of experiments showed that intracellular expression of a full-length transcript generated infectious Sendai virus regardless of whether the plus-sense or the minus-sense RNA was transcribed (41). Success appears to have come from fine tuning the system in terms of the concentration of the polymerase components (N, P, and L proteins) and from constructing plasmids giving rise to transcripts with 5′ and 3′ ends identical to those of the wild-type RNA. Optimization of the system also involved the use of the vaccinia virus inhibitors, cytosine arabinoside and rifampicin. These compounds reduced the cytotoxicity of vaccinia virus and resulted in a dramatic increase of the expression levels of a Sendai virus RNA-like reporter gene. Most interesting was the finding that recovery of infectious Sendai virus was also possible by transfecting naked RNA. The efficiency of recovery appeared to be lower using plus-sense RNA than the genomic minus-sense RNA (41). The latter results involving the use of naked RNAs extend the earlier findings that transfection of naked model RNAs alone results in the efficient amplification and expression of these minigenes in cells infected with Sendai virus (31), respiratory syncytial virus (42) or parainfluenza virus 3 (43, 44). In the future, improvements in the transfection systems to generate novel viruses with ease will provide us with even better tools for the study of negative-strand RNA viruses.

Perspective

The ability to genetically alter negative-strand RNA viruses has already enhanced this field of virology and may have a major influence on future developments in vaccines, gene therapy, cancer treatment, and manufacture of biologicals. First, structure–function studies of individual viral genes are now possible in the context of an infectious virus for a number of negative-strand RNA virus families. These groups consist of many medically important viruses including measles, mumps, respiratory syncytial, parainfluenza, influenza, and bunyaviruses. In the recent past, we tried to take a reductionist approach in virology; viral genes were studied in isolation by cloning and expressing them in different systems. The pendulum has now swung back in the other direction as we ask questions about how viral genes and gene products interact with host cell components and the host in general. This can best be done by studying genetically defined viruses and subjecting them to directed mutational analysis. These viral constructs are then available for biochemical analysis as well as for studying replication and growth in tissue culture or experimental animals. Obviously, structure–function studies of viral genes also include the analysis of promoter elements and other noncoding sequences.

Second, genetically engineered negative-strand RNA viruses should become candidates for use as live virus vaccines. Genetically engineered influenza viruses with changes in coding or noncoding sequences may induce immune responses which are longer-lasting and more protective than those generated by conventional influenza virus vaccines. In the case of respiratory syncytial and parainfluenza viruses, a recombinant DNA approach may be the only rational strategy, since the Jennerian approach has not resulted in acceptable vaccine candidates. Thus, tools are now available to design a new generation of vaccines for the medically important negative-strand RNA viruses.

Third, negative-strand RNA viruses may become useful vectors for the expression of foreign genes. Recombinant influenza viruses (16), rabies viruses (45), and VSV (37) have been used to express additional protein sequences or foreign genes. Packaging limitations and restrictions due to the length or the nature of the foreign gene are not yet defined for negative-strand RNA virus constructs, nor do we have sufficient information about the stability of these viruses once their genome structures have been extensively altered. These uncertainties notwithstanding, there is a major advantage in the use of negative strand RNA viruses as vectors (or as vaccines). These viruses do not go through a DNA phase and thus cannot transform cells by integrating their genetic information into the host cell genome. Furthermore, homologous recombination has never been observed for any of the negative-strand RNA viruses. Thus, replication-incompetent viral constructs grown in complementing cell lines should be free of contaminating virus generated by a recombinational event. In terms of safety, these properties weigh heavily in favor of negative-strand RNA virus vectors.

Novel viruses expressing foreign genes may serve prophylactically as vaccines, or they may play a role in gene therapy when a transient expression would be beneficial. The latter may be the case in cancer therapy, which could require a targeted infection followed by the induction of a lethal cytopathic effect. Repeated administration of negative-strand RNA viruses may not be feasible in this situation because of the host's immune response. However, the choice of different

antigenic variants (as is possible with influenza viruses) may overcome this limitation.

Finally, the highly efficient expression of viral and foreign proteins via negative-strand RNA virus vect rs may have additional bi technological applications. It is possible that defective RNA constructs could be used for genetic immunization. This form of vaccination would resemble DNA vaccination (46) in that the defective particle would go through many replication rounds and persist without spreading to neighboring cells. Such RNA replicons may have interesting biological properties since the efficiency of infection should be comparable to that of whole viruses. Also, replication competent viral vectors may help in the manufacture of large quantities of biological reagents, since the quantities expressed by negative-sense RNA viruses can be high. It is also possible that purification of expressed proteins could be made easier if they were incorporated into extracellular virus particles.

The solutions to many of the issues discussed here will depend on the continuing success of basic science and the development of novel strategies to study viruses. Our horizons must expand and include the analysis not only of the viruses but also of their interactions with the host cell. Only by continuing to study these fundamental processes may we hope to reap the benefits offered to us by these new opportunities.

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The viri n glycoproteins of Eb la viruses are enc ded in two reading frames and are expressed through transcriptional editing

(filovirus/glycoprotein gene/phylogenetic analysis)

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ABSTRACT **In late 1994 and early 1995, Ebola (EBO) virus dramatically reemerged in Africa, causing human disease in the Ivory Coast and Zaire. Analysis of the entire glycoprotein genes of these viruses and those of other EBO virus subtypes has shown that the virion glycoprotein (130 kDa) is encoded in two reading frames, which are linked by transcriptional editing. This editing results in the addition of an extra nontemplated adenosine within a run of seven adenosines near the middle of the coding region. The primary gene product is a smaller (50–70 kDa), nonstructural, secreted glycoprotein, which is produced in large amounts and has an unknown function. Phylogenetic analysis indicates that EBO virus subtypes are genetically diverse and that the recent Ivory Coast isolate represents a new (fourth) subtype of EBO virus. In contrast, the EBO virus isolate from the 1995 outbreak in Kikwit, Zaire, is virtually identical to the virus that caused a similar epidemic in Yambuku, Zaire, almost 20 years earlier. This genetic stability may indicate that EBO viruses have coevolved with their natural reservoirs and do not change appreciably in the wild.**

The 1995 epidemic of Ebola (EBO) virus disease in Zaire (1), coupled with the discovery of an EBO virus in the Ivory Coast in late 1994 (2), has sparked new scientific and public interest in these mysterious and highly pathogenic viruses. EBO viruses are nonsegmented negative-strand RNA viruses that are genetically related to, but distinct from, the Marburg (MBG) virus (3). These viruses, classified in the genus *Filovirus* in the family Filoviridae, cause a severe hemorrhagic disease in human and nonhuman primates (4, 5). The EBO group of filoviruses comprises three discrete subtypes: Zaire, Sudan, and Reston (EBO-Z, EBO-S, and EBO-R, respectively). In late November 1994, EBO virus was discovered in the Tai Forest of the Ivory Coast (1). This virus (EBO-IC) caused a single nonfatal human infection and was believed to have caused increased mortality in a troop of wild chimpanzees. Preliminary serological analysis of the EBO-IC isolate suggests that it may represent a fourth subtype (ref. 2; Centers for Disease Control and Prevention, unpublished data). The much publicized outbreak of human disease in the city of Kikwit, Zaire, in late Spring, 1995, involved over 300 cases with a 77% mortality rate (1, 6). This epidemic was caused by a strain of EBO-Z, the most pathogenic subtype of EBO virus, which had not been reported for 18 years.

The virion surface glycoproteins (GPs) of filoviruses are multimers of a single structural GP; they are important in the binding of virions to cell receptors and virus entry into the cell cytoplasm (3). This protein is expressed from the GP gene, which in the genomes of filoviruses is positi ned fourth (from the 3′ end) of seven linearly arranged genes (3, 7). Filovirus GPs are highly glycosylated, containing both N-linked and O-linked carbohydrates (3, 8–10) that contribute from one-third to one-half of their relatively large molecular weight (M_r ≈130–170 kDa).

To better define the relationship of EBO viruses to one another, we have investigated the structure and expression of their GP genes.† Here we describe an unusual organization of the GP genes of all EBO virus subtypes, present a phylogenetic profile for the family Filoviridae, and discuss the role of GP gene products in the pathogenesis of EBO virus disease.

MATERIALS AND METHODS

Viruses. The initial EBO-R isolate (R/USA/Reston/1989/119810 strain) was obtained from P. B. Jahrling (U.S. Army Medical Research Institute for Infectious Diseases, Fort Detrick, MD) and was isolated and passaged once in MA-104 cells, plaque purified three times, and passaged once in Vero E6 cells. Two 1992 EBO-R viruses [R/Philippines/Manila/1992/920084, isolated at Centers for Disease Control and Prevention (Atlanta), and R/Italy/Siena/1992/12552, obtained from D. Brown (Public Health Laboratory Service, Virus Reference Laboratory, London)] were passaged 2 to 3 times in Vero E6 cells and once in MA-104 cells. A 1976 EBO-Z virus (Z/Zaire/Yambuku/1976/057935 also referred to as the Mayinga isolate) was passaged once in Vero cells and once in Vero E6 cells. The passage histories of other EBO-Z viruses (Z/Zaire/Yambuku/1976/057878 and Z/Zaire/Tandala/1977/088296, also referred to as the Eckron and Bonduni isolates, respectively) and EBO-S viruses (S/Sudan/Maridi/1976/VCP2D11 and S/Sudan/Nzara/1979/015176, also known as the Boneface and Maleo isolates, respectively) are described elsewhere (11). The EBO-IC (IC/Ivory Coast/Tai Forest/1994) was obtained from B. Le Guenno (Institute Pasteur, Paris), and was passaged 2 to 5 times in Vero E6 cells. The EBO-Z virus from the 1995 epidemic in Kikwit (Z/Zaire/Kikwit/1995/9510621) was isolated at the Centers for Disease Control and Prevention in Vero E6 cells inoculated with a blood specimen from an acutely infected patient (who later died). However, all sequence data related to this strain were derived from RNA extracted from the same human blood specimen.

Viral RNA Purification, Amplification, and Cloning. Preparation of genomic RNA (vRNA) from purified EBO virions and the extraction of total infected cell RNA were performed as described (12). Syntheses of cDNA from the vRNA of EBO-R (Reston/1989) and EBO-S (Nzara/1979) were performed (13), followed by blunt-end ligati n into the *Sma* I site

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Abbreviations: EBO, Ebola; MBG, Marburg; TCF, tissue culture fluid; GP, glycoprotein; SGP, small/secreted glycoprotein precursor; RT-PCR; reverse transcription–PCR; PMSF, phenylmethylsulfonyl fluoride; ORF, open reading frame; RIP, radioimmunoprecipitation.

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†The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U23069, U23152, U23187, U23416, U23417, U28006, U28077, and U28134).

of pUC18 or pSP73/pSP72 vectors (Promega) and cloned as previously described (14). Sequencing of cloned plasmid preparations was performed using the dideoxy chain termination method (15). A manual radiolabeling method employing cloned bacteriophage T7 DNA polymerase (Sequenase) and an automated nonisotopic method (dye-terminator cycle sequencing; Perkin–Elmer) was used. Direct (automated) sequencing of DNA amplified from viral sequences by reverse transcription–PCR (RT-PCR) was used in sequencing the EBO-S strains, EBO-IC, the Kikwit/1995 strain of EBO-Z, and the 1992 EBO-R isolates. RT-PCR amplification of purified vRNA sequences was performed using GenAmp RNA PCR kits (Perkin–Elmer) or reactions were assembled using reagents and buffers obtained from commercial sources (Promega and Boehringer Mannheim).

Quantitation of Transcriptional Editing. Editing of GP gene transcripts was quantitated for EBO-R (Reston/1989) and EBO-Z (Yambuku/1976/057935) by RT-PCR amplification of GP sequences from crude infected cell RNA (mRNA) preparations. First-strand cDNA synthesis was performed by heating approximately 5 μg of total infected cell RNA and 300 ng of the primer 5′-CCGGTACC$(T)_{35}$ at 65°C for 1 min and placing the mixture at 42°C; the remaining components were then added (25-μl reaction volume). Primer extension with reverse transcriptase proceeded for 40 min, followed by adding 100 μl of sterile water and boiling for 3 min. Approximately 5–10 μl of this diluted reaction solution served as template for the amplification of viral sequences. For PCR amplification of the EBO-R GP gene editing region, the primers 5′-CCTTAGCAACAGTACAGGGAGAT and 5′-GCCGTCTCTTGCGGGTCCTGG were used. For the EBO-Z GP gene, the primers 5′-CTGGATCCAACAACACAATGGGCGTTACAGGAAT and 5′-AATGGTACCGACTCGTGGAGATTGTGG were used to amplify the editing region. The EBO-R PCR product was cleaved with the restriction enzyme *Bam*HI and ligated into the *Bam*HI site of pUC18. The EBO-Z PCR product was cleaved with *Kpn* I followed by *Bam*HI, directionally ligated into pUC18, and isolated clones sequenced.

To determine if virion (genomic) RNA might contain eight or more uridines at the editing site, vRNA was extracted from a human blood specimen (from which the Kikwit/1995 virus was isolated) and used as template in a RT-PCR to amplify a DNA fragment containing the editing site. The primer 5′-GGACCCGTCTAGTGGCTACTATTC (plus-sense) was used to prime first-strand cDNA synthesis from vRNA template molecules, as above, then the reaction was boiled 3 min before its use in PCR amplification. The above primer was then used with the negative-sense primer 5′-CCAATGCATGATGCGACACTGCAGCTTCCC (nonviral sequences are underlined) to amplify a 482-bp fragment containing the editing region. The RT-PCR product was isolated by agarose gel electrophoresis and extracted from the gel using a commercial kit [QIAEX II, Qiagen Inc. (Chatsworth, CA)]. Isolated DNA was directly ligated into the plasmid pCRII using a TA prokaryotic cloning kit (Invitrogen). Cloned plasmid DNA was isolated and individual clones were sequenced.

***In Vitro* Expression of EBO-R GP Gene Products.** *In vitro* expression of EBO-R (Reston/1989) GP gene sequences was performed by RT-PCR amplification of the GP open reading frame (ORF) from total infected-cell RNA using the primers 5′-ATACCCGGGCCCCAAATTACCTATACAACA and 5′-TTTTCTAGAATATTAATCATTATTAAGAGA. Amplified products were digested with *Xma* I and *Xba* I and directionally ligated into pSP73, cloned, and full-length inserts containing seven or eight adenosines in the editing site were isolated (pSP73-RESGP-7A and pSP73-RESGP-8A, respectively). Plasmids were linearized with *Hin*dIII transcribed with SP6 RNA polymerase, DNase-treated, and run-off transcripts were *in vitro* translated using a rabbit reticulocyte lysate system (Promega).

Radioimmunoprecipitation (RIP) of EBO Virus GPs. Monolayers of MA-104 cells in 24-well panels were infected with EBO viruses (≥10 plaque-forming units per cell) or mock infected. Cells were incubated for 3 to 4 days, washed with Dulbecco's minimal essential medium deficient in methionine and/or cysteine containing 2% dialyzed fetal bovine serum and antibiotics, and 300 μl of the same medium plus 20 μCi/ml [^{35}S]methionine/cysteine. Cells were incubated for 4 hr, then tissue culture fluid (TCF) was removed and the cells were washed as above. The cell monolayer and 900 μl of harvested TCF were treated with 100 μl 1% SDS for 1 min, followed by the addition of 900 μl 1.1× TNE buffer (1× TNE = 10 mM Tris·HCl/150 mM NaCl/3 mM EDTA, pH 7.6) containing 1.1% Nonidet P-40, 0.55% sodium deoxycholate, and 1.1 mM phenylmethylsulfonyl fluoride (PMSF). Triton X-100 treatment was performed by the addition of 1× TNE containing 1% Triton X-100 and 1 mM PMSF to cells; TCF was made to 1% with Triton X-100 and to 1 mM PMSF. RIP assays were performed on 500 μl of solutions as described (14). Antibodies used in RIP assays were: (*i*) an anti-EBO-R GP/small/secreted glycoprotein precursor (SGP) monoclonal antibody (high-titered mouse ascitic fluid; cross-reactive with GP and SGP) prepared essentially as described (16), and (*ii*) a high-titered polyclonal mouse anti-EBO-Z GP produced against a GP expressed by a recombinant baculovirus (10). Endoglycosidase digestion of RIP products was performed as described elsewhere (17).

Computer-Aided Sequence Analyses. Computer analyses of nucleic acid and predicted amino acid sequences for the GP genes of EBO subtypes were performed using the Genetics Computer Group (Madison, WI) Sequence Analysis Software Package (Version 7.3-AXP) run on a Digital AXP Alpha Workstation. The PILEUP program was used to align nucleotide sequences with a GapWeight setting of 5.0 and a GapLength-Weight setting of 0.5. Phylogenetic analysis of nucleotide alignments was performed using the PAUP software Version 3.1.1 (developed by D. L. Swofford) run on a Power Macintosh model 8100/110.

RESULTS

The sequences of the entire GP genes of EBO-Z, EBO-S, EBO-R, and EBO-IC were determined from cloned cDNA and RT-PCR amplified DNA products (see Fig. 1 legend for GenBank accession numbers). The EBO-IC strain was found to differ substantially from the other subtypes of EBO virus (≤60% identity) and represents a fourth subtype. The organization and specific features of these genes are shown in Fig. 1. The most unusual finding was that the GPs of all EBO subtypes are encoded in two frames and that in each case an SGP was predicted as the primary gene product.

The GP genes of all EBO viruses begin and end with conserved transcription start and stop (polyadenylylation) sites (14). The only difference in GP gene organization seen in the four subtypes is found in EBO-R. For the other subtypes, the transcription stop site overlaps the transcription start sites of the downstream (VP30) gene and is centered on the common pentanucleotide sequence 3′-UAAUU found in all transcriptional signals (3′-CUACUUCUAAUUCUUUUU). In contrast, the GP gene stop site of EBO-R is separated from the downstream gene by a short intergenic region of three nucleotides that is followed by a conserved start site (3′-UAAUUCUUUUU-GAA-UACUGCUUCUAAUU).

The SGP and GP share the same N-terminal ≈300 residues, but have unique C-terminal sequences (Fig. 2). Unlike that of the GP, the C terminus of the SGP lacks a transmembrane anchor sequence, which results in its secretion from infected cells. The unique C-terminal portion of the SGP is hydrophilic and rich in charged residues (mostly positive). The entire SGP sequence for EBO subtypes is relatively conserved, but the central area of the GP contains a large variable hydrophilic

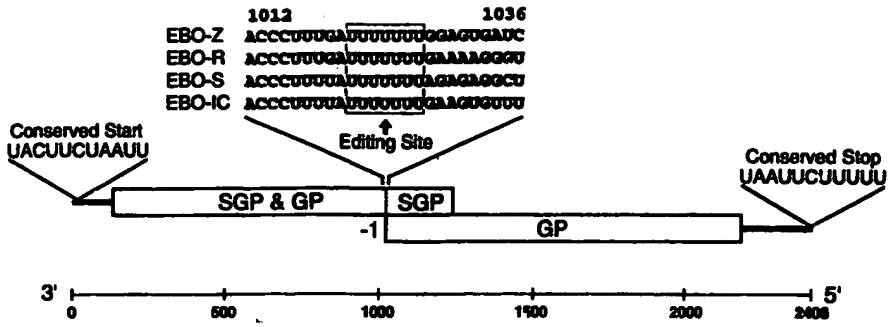

FIG. 1. Schematic representation of the GP gene organization for the EBO-Z, EBO-R, EBO-S, and EBO-IC subtypes. GenBank accession numbers for EBO virus sequences are U23187 (Yambuku/1976/057935), U28077 (Kikwit/1995), U23152 (Reston/1989), U23416 (Manila/1992; ORF only), U23417 (Siena/1992; ORF only), U28134 (Maridi/1976), U23069 (Nzara/1979), and U28006 (Tai Forest/1994). Shown are the conserved transcriptional start and stop signals, coding regions, and the site of transcriptional editing. An alignment of the region containing the transcriptional editing sites for the four subtypes of EBO virus is also shown (EBO-Z sequence shown = 1012–1036). All sequences are shown in the minus sense. The scale at the bottom represents the length of the EBO-Z GP gene (2408 nucleotides).

region (Fig. 2) where more than half of the potential N-linked glycosylation sites and the putative region for O-linked glycosylation are located. The rest of the GP sequence is conserved and is more hydrophobic. In addition, all the cysteine residues in the SGP and GP are conserved (see asterisks in Fig. 2). One common feature found in the GPs of all filoviruses is a highly conserved immunosuppressive motif (26 residues) in the C-terminal third of the GP that has a high degree of homology to those found in the envelope glycoproteins of oncogenic retroviruses (9, 18).

Since sequence analysis indicated that the GPs of EBO viruses are encoded in two frames, it was important to determine the mechanism(s) by which the GP is expressed. The original published description of the EBO-Z (Yambuku/1976/057935) GP gene (7) was based on a virus stock that had undergone three rounds of plaque purification and was later determined to contain a single base insertion (extra uridine nucleotide) that joined the GP frames. Because a single base insertion led to the connecting of the frames in the mutant (plaque purified) EBO-Z, it was thought that RNA editing at or near this site, in a manner similar to that described for the P genes of paramyxoviruses (19), may be responsible for the generation of GP. This region of the EBO-Z (Yambuki/1976/057935) and EBO-R (Reston/1989) GP mRNA were RT-PCR amplified and cloned, and the isolated clones were sequenced. From a total of 81 EBO-R and 41 EBO-Z clones, an addition of a single nontemplated adenosine in the mRNA at the suspected site was found in 13 (16%) and 11 (27%) of the clones, respectively. Fig. 1 shows a short alignment of the GP gene region containing the editing site for the four subtypes of EBO virus. To eliminate the possibility that the insertion of an extra adenosine merely reflects template (vRNA) variability, RT-PCR was directed at negative sense EBO-Z (Kikwit/1995) RNA isolated from a human blood sample. Sequencing of 32 clones showed no insertions in the template vRNA at the editing site, indicating that the genomic sequence is homogeneous and transcriptional editing is responsible for the heterogeneity seen in the GP mRNA clones.

To confirm that the synthesis of SGP and GP are directed by unedited and edited transcripts, respectively, *in vitro* expression of the EBO-R (Reston/1989) GP ORF was performed. Two plasmids were used to generate run-off transcripts, one that contained seven adenosines at the editing site (mRNA sense) and one that had eight adenosines (GP frames connected). Products of *in vitro* translation of run-off transcripts are seen in Fig. 3*A*. Translation of the RNA containing seven adenosines (unedited form) resulted in the synthesis of an unglycosylated protein that is close to the predicted size of the SGP backbone (41.8 kDa) (Fig. 3*A*, lane 1). This protein was processed to a higher molecular weight when the transcript was translated in the presence of canine pancreatic microsomal membranes (lane 2). Similarly, the RNA with eight adenosines (edited form) produced a prominent protein in the predicted size range for the unglycosylated GP and could also be processed to a higher molecular weight form (lanes 3 and 4). Translation of the transcript with seven adenosines produced not only SGP, but also a small amount of unglycosylated GP that was processed in the presence of membranes. Synthesis of GP from the unedited transcript may have occurred via translational frameshifting in a −1 direction (20, 21), or may have occurred due to addition of an extra nucleotide at the editing site (or some other site) by the SP6 RNA polymerase, similar to the transcriptional editing carried out by the EBO virus polymerase. The processed forms of SGP and GP expressed *in vitro* correspond in size to SGP and GP immunoprecipitated from the cells and TCF of EBO-R-infected MA-104 cultures (Fig. 3*A*, lanes 5–8). The SGP produced *in*

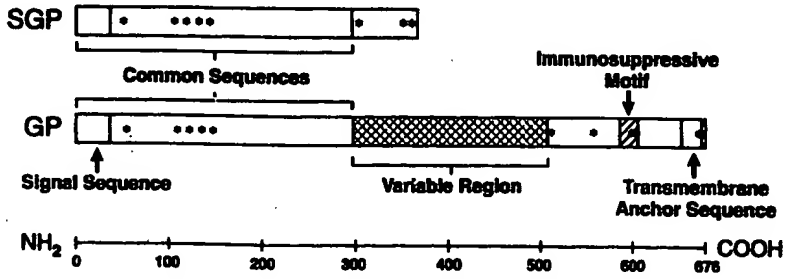

FIG. 2. Profiles of SGP and GP amino acid sequences of EBO subtypes. SGP and GP share the same N-terminal ≈300 residues. Identified on the schematic drawing are the signal sequences for the SGP and GP, the GP C-terminal transmembrane anchor sequence, and an immunosuppressive motif found in the GP. Conserved cysteine residues are identified by asterisks. A highly variable central region of the GP is identified, while all other regions are relatively conserved. The lengths of the SGP and GP for the EBO-Z subtype are 364 and 676 amino acids, respectively.

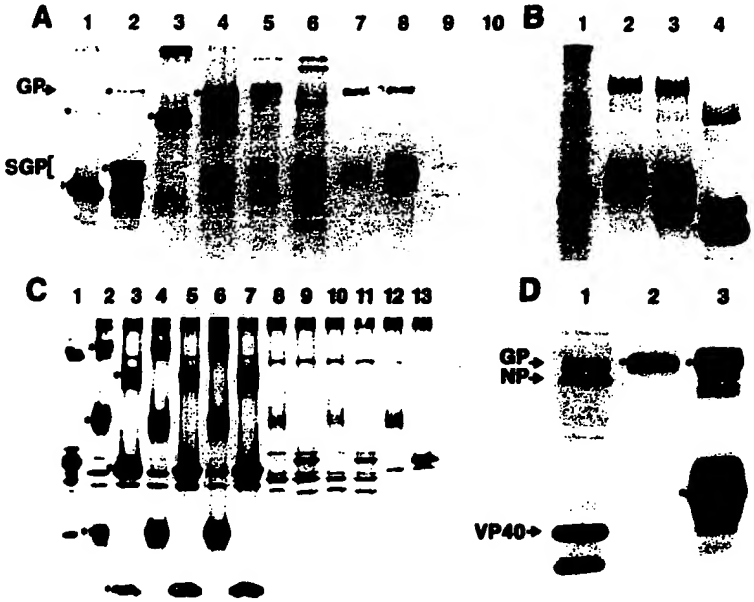

FIG. 3. Expression of EBO virus GPs. (*A*) Fluorogram of [^{35}S]methionine/cysteine-labeled EBO-R GPs expressed *in vitro* and *in vivo* separated on SDS/polyacrylamide gels (10% gel). *In vitro* translation products of run-off transcripts generated from the plasmid pSP73-RESGP-7A (lanes 1 and 2) and pSP73-RESGP-8A (lanes 3 and 4), each pair without and with addition of canine pancreas membranes, respectively. Asterisks next to lanes 1–4 identify the unprocessed and larger processed (glycosylated) forms of SGP and GP. The remaining lanes contain RIP products (bound by an anti-EBO-R GP/SGP monoclonal antibody) from EBO-R (Reston/1989)-infected MA-104 cell lysates (lanes 5 and 6), supernatant fluids (lanes 7 and 8), or mock-infected cell supernatant fluids (lanes 9 and 10). Lanes 5, 7, and 9 contain products derived from lysates prepared by SDS treatment (followed by Nonidet P-40 and deoxycholate), and lanes 6, 8, and 10 were treated with Triton X-100 (13). At the left edge are marked the positions where GP and the broader SGP bands migrate. Asterisks next to lane 8 identify SGP and GP immunoprecipitated from supernatant fluids. (*B*) Endoglycosidase treatment of the EBO-R GP and SGP. Lane 1 (marker lane) contains the same *in vitro* translation products as lane 1 in *A*. The same preparation separated in lane 8 in *A* was mock-treated (lane 2) or digested with either endoglycosidase H (lane 3) or endoglycosidase F/N-glycosidase F mixture (lane 4). Asterisks identify digestion products. (*C*) Immunoprecipitation of SGP and GP from EBO virus-infected cell supernatant fluids. Lane 1 (marker lane) contains EBO-Z (Yambuku/1976/057935) virion proteins. The remaining lanes are paired (2 and 3, 4 and 5, etc.) and contain mock-treated (even lanes) and endoglycosidase F/N-glycosidase F-treated (odd lanes) proteins immunoprecipitated with a mouse polyclonal anti-EBO-Z GP. RIP products were derived from the supernatant fluids of MA-104 cells infected with three different EBO-Z (lanes 2–7; viruses Yambuku/1976/057935 and 057878, and Tandala/1977, respectively), two EBO-S (lanes 8–11; viruses Nzara/1979 and Maridi/1976), and EBO-R (lanes 12 and 13; Reston/1989). Asterisks identify three prominent GP bands and deglycosylated products seen in EBO-Z lanes. (*D*) Demonstration that the SGP of EBO-R is a nonstructural GP. Purified virion preparations, labeled with either [^{35}S]methionine (lane 1) or [^{3}H]glucosamine (lane 2), are shown together with the same RIP products as lane 8 in *A*. Asterisks identify the virion GP in lanes 2, and the GP and SGP in lane 3. At the left edge are marked the locations of the virion GP, nucleoprotein (NP), and VP40 (matrix protein).

vitro, however, did not appear as broad (heterogeneous) as the SGP detected in the TCF of EBO-R-infected cultures.

The secreted EBO-R SGP ranges in size from 50 to 70 kDa, with the central major band estimated to be 59 kDa. Endoglycosidase digestion indicated that at least three major species are present (Fig. 3*B*). Removal of all N-linked glycans by Endo F/NF produced two bands, a smaller predominant species and a weaker larger band that may contain O-linked glycans or represents some other form of the SGP. The SGP found in the supernatant fluids of cells infected with EBO-Z and EBO-S are comparable with those seen with EBO-R (Fig. 3*C*). The EBO-Z strains showed strong bands that correspond to the GP and SGP described above for EBO-R, but cross-reactivity of the anti-EBO-Z GP with the EBO-S and EBO-R subtypes resulted in much weaker and thinner bands. Surprisingly, the Zaire subtypes produced a third glycosylated protein band (≈24 kDa) of unknown origin. Fig. 3*D* shows that the SGP of EBO-R is absent from purified virion preparations (as is the case for the other EBO subtypes) and is thus a nonstructural protein. As with the SGP, the smallest GP noted above has not been detected in virions.

The EBO virus GP genes differed from those of MBG virus by at least 55% at the nucleotide level and 67% at the amino acid level. Even among the EBO subtypes a high degree of genetic variability was evident. Four clearly distinct EBO virus subtypes (Zaire, Sudan, Reston, and Ivory Coast) were identified, differing from one another by 37–41% and 34–43% at the nucleotide and amino acid level, respectively. A significant finding was that the nucleotide sequence of the Kikwit/1995 EBO-Z differed from the Yambuki/1976/057935 isolate by <1.6% over the entire gene. In addition, no amino acid sequence differences are predicted for the SGPs of these viruses, but a 2% difference in the GP was noted, primarily in the variable region where 11 of the 13 amino acid changes occurred.

A detailed phylogenetic profile of the family Filoviridae was determined using nucleotide sequences that encode N- and C-terminal regions of the GP. These regions contained sufficient homology to allow accurate nucleotide alignment. Maximum parsimony analysis of these sequences produced a single most parsimonious tree that clearly separated EBO and MBG viruses and divided the EBO subtypes into four distinct clades (Fig. 4).

DISCUSSION

The finding that all EBO subtypes encode their structural (virion) GPs in two frames and that expression of these GPs occurs through transcriptional editing is highly unusual. Our results indicated that there was no sequence variation in the EBO vRNA corresponding to the mRNA editing site when analyzed directly from the blood of an acutely infected human.

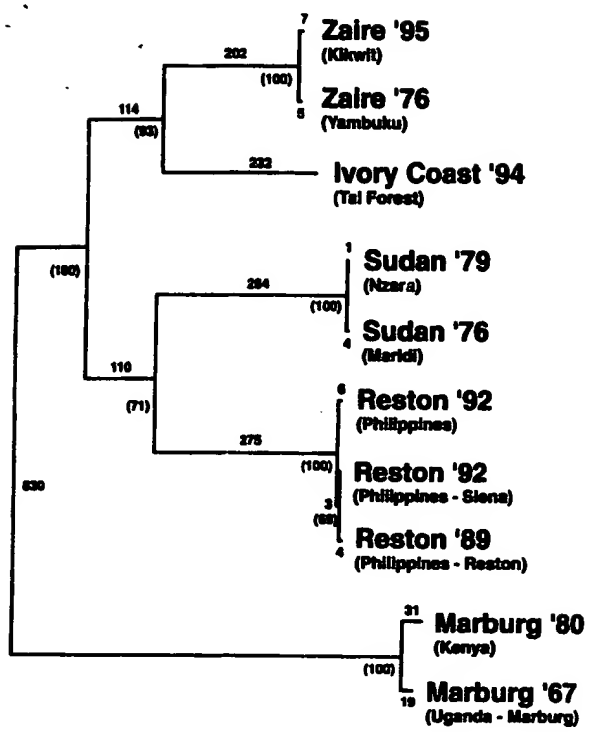

FIG. 4. Phylogenetic relationship of filovirus GP gene sequences. Shown is a phylogenetic tree obtained from an alignment of concatenated nucleotide sequences that encode conserved N-terminal and C-terminal regions of filovirus GP ORFs. The ORF sequences for EBO-Z, EBO-IC, and EBO-S are nucleotides 46–536 + 1528–1885, for EBO-R 49–539 + 1531–1888, and for MBG 2–489 + 1532–1589 (1 = start of ORF). Maximum parsimony analysis (branch-and-bound with a 4:1 weighting of transversions to transitions) produced a single most parsimonious tree. The values for bootstrap confidence limits (%) for branch points were generated from 1000 replicates of the analysis and are indicated in parentheses at branch points. GenBank accession numbers for sequences used in phylogenetic analyses are U28077, U23187, U28006, U23069, U28134, U23416, U23417, U23152, Z12132, and Z29337.

This reinforces our belief that the encoding of the GP in two frames may be important in the maintenance of EBO in nature and perhaps also in the pathogenesis of human disease. We have no data bearing on the consequences of the circulation of a soluble protein bearing the N-terminal 300 amino acids of the virion GP, and we have not found homologues of the novel SGP sequences (C-terminal 70–77 residues) that suggest a specific role in the infectious process. We speculate that the SGP may interact with the immune system, either at the cellular level, where the result could be cellular deletion, anergy, or activation of suppression, or at the humoral effector level, where high-affinity antibodies directed against the N-terminal portion of the GP may be prevented from acting on virions or cells presenting surface GP. Indeed, patients usually die without evidence of an effective immune response, and even when recovery is underway survivors do not have detectable virus neutralizing antibodies. Our preliminary investigations have shown that SGP is detectable in high concentrations in the blood of acutely infected patients (data not shown). If the SGP does play an important role in the pathogenesis of human infection, it may be possible to protect or increase survival rates if it can be targeted by specific antibodies early in the infection.

The discovery of a third and even smaller 24-kDa glycoprotein in the medium of EBO-Z-infected cells was unexpected. This protein, which is reactive with an anti-EBO-Z GP serum, may result from cleavage or degradation of GP or SGP or may be produced as a result of a premature termination event during translation. Further studies are required in order to determine how this protein is produced in infected cells and if this protein is unique to certain EBO subtypes.

The immunosuppressive motif identified in the C-terminal third of filovirus GPs is the most conserved sequence seen between the GPs of EBO and MBG viruses (7), and may contribute to the immunosuppression observed in humans and monkeys infected with filoviruses. In addition, this motif may have a role in the assembly of the GP into peplomers. Immediately upstream and slightly overlapping the N-terminal end of the immunosuppressive motifs of all filovirus GPs is a region that contains a heptad repeat sequence that Chambers *et al.* (22) identified in the fusion GPs of oncogenic retroviruses (the same location with respect to the immunosuppressive motif of p15E) and paramyxoviruses. They postulated that these repeats may form an extended backbone for the spike structures through the coiling of the GPs around one another at these regions.

Phylogenetic analysis of GP gene nucleotide sequences clearly distinguishes the EBO subtypes from MBG virus, but also indicates that each of the four EBO subtypes represents a monophyletic lineage. The close phylogenetic relationship of EBO-R strains, filoviruses associated with monkeys exported from the Philippines (23–25), to the African subtypes may indicate that this EBO-R is not indigenous to Asia but may have been introduced to this region from Africa.

The similarity in the GP genes of the 1976 and 1995 EBO-Z strains (<1.6% for the entire gene) is surprising, since more than 18 years and 1000 km separate the outbreaks caused by these viruses. These data may indicate that EBO viruses (and filoviruses in general) have evolved to occupy relatively stable specific niches in nature and may not change appreciably in their natural hosts. The high degree of similarity between the 1976 and 1995 EBO-Z viruses suggests that the reservoir is the same in both locations and that it is widespread in Zaire or is a migratory species. In contrast, the divergence seen among the different EBO subtypes and between EBO and MBG viruses might also imply that these viruses are very old and have slowly coevolved with their as-yet-unknown natural hosts, which may also demonstrate a similar extent of genetic variability. While the genetic diversity among the four EBO virus subtypes is high, the apparent stability of each subtype bodes well for the development of effective immune or antiviral therapy or vaccination strategies. A large scale effort is currently underway to attempt to discover the natural reservoir for EBO viruses. This includes screening of large numbers of vertebrates and invertebrates from the Kikwit area for evidence of EBO virus infection. Currently, we have insufficient knowledge to help narrow the focus of this search, although considerable effort is being placed on vertebrates in the collection. This decision is influenced by the genetic stasis observed between the EBO-Z strains from 1976 and 1995, and by the fact that all filoviruses examined possess an immunosuppressive domain in their GP that to date has only been found in vertebrate RNA viruses.

In conclusion, we have described an unusual type of organization for the GP genes of all EBO viruses, which has not been described for any other type of virus gene encoding a structural GP. We feel that the expression of this gene is an important element in the pathogenesis of EBO viruses and is critical to their maintenance in the natural host. In the future we plan to continue studying the expression of EBO virus GPs *in vitro* and *in vivo*, to examine the roles of SGP and GP in the disease process, and to determine if these proteins can serve as protective immunogens.

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